Optimization of the Firefly Luciferase Assay for ATP[1]

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The conditions for maximum light emission in ATP analyses using firefly lantern extracts, partially purified luciferase preparations, and crystalline luciferase were determined. Tricine buffer (pH 7.8) used at a final concentration of 0.025 M was found to be the best of several buffers tested. Mg^{2+} was required and its optimum concentration was 5 mM. The relative amounts of luciferase and luciferin influenced light output; for maximum light production the luciferase preparations should be supplemented with extra luciferin. Under normal assay conditions oxygen was not limiting. Bovine serum albumin, EDTA, and dithiothreitol were all found to stabilize luciferase and yield more reproducible results. Modification of the sample holder for the SAI Model 3000 photometer and the use of 0.2 ml reaction volume increased the measured light output. ATP standard graphs constructed by using chart recorder peak height, instrument peak height, and integration mode determination of light output consisted of parallel lines. The conditions for reagent stability were defined.

INTRODUCTION

The availability of commercial firefly luciferase reagents and of instrumentation for measuring light production is markedly increasing the analytical application of bioluminescence. Three symposia have been devoted to determination of ATP and the analytical application of bioluminescence and chemiluminescence (1–3). Several methods of luminescent analysis were described in Methods in Enzymology, Vol. 57 (4), and a recent monograph (5) has provided a comprehensive treatment of bioluminescence. Webster *et al.* (6) have compared several characteristics of the commercially available firefly luciferase preparations. In this paper we report the results of experiments aimed at optimization of the firefly luciferase assay of ATP using commercially available equipment and reagents.

MATERIALS AND METHODS

Enzymes and chemicals. Luciferase preparations were obtained from E. I. Du Pont de Nemours and Company and from the Sigma Chemical Company. The luciferase from Du Pont (120 mg dry wt) was dissolved in 3 ml of the appropriate buffer (0.05 M, pH 7.8) containing 10 mM $MgSO_4$, 1 mM EDTA, and 1 mM dithiothreitol. Sigma firefly lantern extract FLE-50 was reconstituted in 5 ml of water to give a solution (pH 7.4) containing 0.05 M potassium arsenate and 0.02 M $MgSO_4$. Sigma Type IV luciferase (crystalline, 1 mg) was dissolved in 200 μl of 10%

TABLE I

Effect of Various Buffers on Luciferase Activity[a]

Buffer[b]	pK_a (20°C)	Activity relative to that in Hepes
Mops	7.20	0.65
Phosphate	7.21	0.09
Tes	7.50	0.54
Hepes	7.55	1.00
Hepps	8.00	0.68
Tricine	8.15	1.25
Glycinamide	8.20	0.80
Tris	8.30	1.00
Bicine	8.30	0.70
Glycylglycine	8.40	0.72

[a] The activity of Du Pont luciferase (400 μg dry wt) was determined in solutions of the indicated buffers of pH 7.8. Reaction mixtures (1 ml) contained 50 ng ATP, 5 mM Mg^{2+}, 0.5 mM EDTA, and 0.5 mM DTT.

[b] Concentration of each, 0.025 M.

ammonium sulfate and then diluted to 1 ml to give a solution containing 0.05 M Tricine,[2] 10 mM $MgSO_4$, 1 mM EDTA, 1 mM dithiothreitol, and 0.1 mg of bovine serum albumin. Luciferin was synthesized by Dr. A. S. Radhakrishna in the laboratory of Dr. K. D. Berlin. Arsenite was obtained from Mallinckrodt, Chelex from Bio-Rad, DTT from Calbiochem, and EDTA from Fisher. Other enzymes and chemicals were purchased from the Sigma Chemical Company.

Light measurement. Light production was measured in a SAI Technology Model 3000 ATP photometer equipped with a Houston Instrument Omni Scribe Model 35217-5 recorder. Using a luminol standard, 1.4×10^4 photons produced the voltage recorded as one count on the digital readout. Thus 1000 counts (1.4×10^7 photons) represents the production of one light unit.

RESULTS AND DISCUSSION

Assay Components

Buffer. Several characteristics of the luciferase reaction mixture influence luciferase activity. Thus, there is an anion-binding site on luciferase which influences enzymatic activity and the wavelength of light emitted from the activated luciferin depends on pH (8). Webster *et al.* (9) have found that firefly luciferase has different conformations that give differences in enzymatic activity depending upon the buffer used. Formerly, arsenate buffer was used by Strehler and Totter (10) to inhibit luciferase and thus slow down the light production so that measurements could be made with the instruments available at that time. McElroy (11) has recently reminded users of firefly luciferase that arsenate is no longer required with

[2] Abbreviations used: Mops, 3-(N-morpholino)propanesulfonic acid; Tricine, N-[tris(hydroxymethyl)methyl]glycine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tes, 2-[tris(hydroxymethyl)methylamino]ethanesulfonic acid; Hepps, 4-(2-hydroxyethyl)piperazine-propanesulfonic acid; Bicine, N,N-bis(2-hydroxyethyl)glycine; DTT, dithiothreitol; BAL, British anti-Lewisite,2,3-dimercapto-1-propanol.
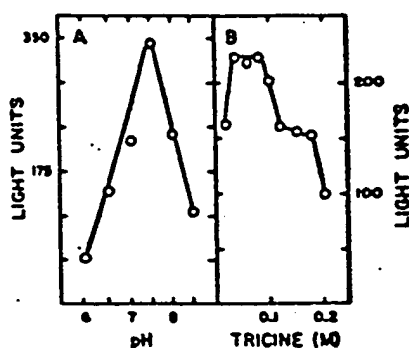
FIG. 1. pH optimum and effect of buffer concentration on luciferase activity. (A) Tricine buffer (0.025 M) at various pH values used with 100 ng ATP, 400 μg Du Pont luciferase-luciferin, 5 mM Mg^{2+}, 0.5 mM EDTA, and 0.5 mM DTT. (B) Various Tricine buffer concentrations of pH 7.8 used. The reaction system (1 ml) contained 100 ng ATP, and 0.1 mM Mg^{2+}.

the current light detectors and fast recorders now available, and can only lower the sensitivity of the assay.

Table I lists several buffers with pK_a values between 7.20 and 8.40 and shows the activity of luciferase in each buffer. Tricine-buffered reaction mixtures yielded the greatest light production. There appears to be no relationship between the pK_a of the buffer and the luciferase activity.

Because of the low enzymatic activity observed in phosphate buffer (possibly due to the presence of heavy metals (11)) both the phosphate and Tricine buffers were treated with Chelex (12). Since there was only a slight increase in enzymatic activity, metal contamination was not considered significant.

The optimum pH for luciferase activity was 7.8 (see Fig. 1A). This optimum is consistent with that observed by Green and McElroy (13). The effect of various buffer concentrations on enzymatic activity is shown in Fig. 1B. With buffer concentrations greater than 0.1 M there was considerable inhibition of luciferase activity. Since firefly luciferase is an euglobulin some salt is required to keep it in solution. Tricine buffer (0.025 M) of pH 7.8 was used in the standard ATP assay system.

Magnesium ion concentration. Mg^{2+} reacts with ATP to form a magnesium ion–ATP complex which is the actual substrate for luciferase (4). The following Mg^{2+} salts were equally suitable: $MgSO_4$, $MgCl_2$, and $Mg(O_2CCH_3)_2$. Figure 2 shows the effect of different Mg^{2+} concentrations on enzymatic activity—a final Mg^{2+} concentration of 5 mM was used.
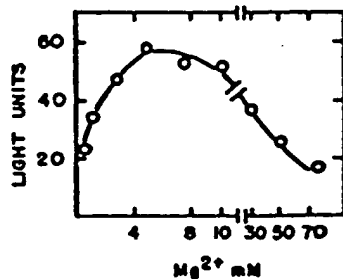

FIG. 2. Effect of Mg^{2+} concentration on luciferase activity. The reaction system (1 ml) contained 400 μg Du Pont luciferase-luciferin, 10 ng ATP, 0.5 mM DTT, and various amounts of Mg^{2+}.
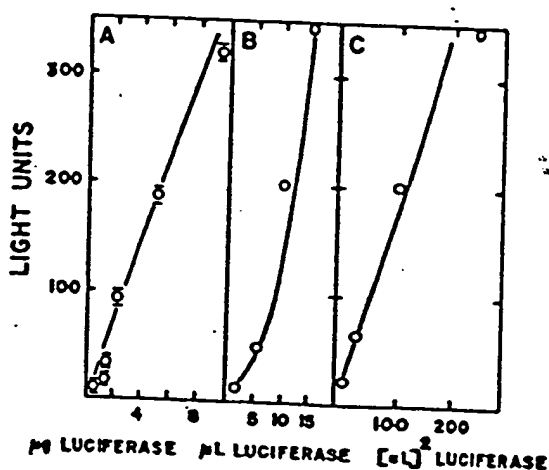
Fig. 3. Effect of luciferase concentration on light production. (A) Various concentrations of Sigma Type IV used in the 1-ml reaction system containing 1 ng ATP, 50 μg luciferin, 0.025 M Tricine, pH 7.8, 5 mM Mg²⁺, 0.5 mM EDTA, and 0.5 mM DTT. (B) Various concentrations of the Du Pont luciferase–luciferin preparation were used in a reaction mixture (1 ml) containing 5 ng ATP, 0.025 M Tricine, pH 7.8, 5 mM Mg²⁻, 0.5 mM EDTA, and 0.5 mM DTT. (C) Data from (B) replotted using as abscissa the square of the volume of luciferase–luciferin preparation used.

Luciferase. Figure 3 shows the light output obtained using various amounts of Sigma Type IV luciferase (A) and Du Pont luciferase (B). Figure 3A shows that the light produced by Sigma Type IV enzyme is proportional to protein concentration. However, the addition of increasing volumes of Du Pont luciferase–luciferin reagent (Fig. 3B) does not result in a linear increase in light production because the amount of luciferin present in the preparation is limiting. Supplementation of the Du Pont luciferase–luciferin-containing reaction mixture with 50 μg of luciferin produced a linear response (data not shown). Figure 3C shows a plot of enzymatic activity against the square of the volume of the Du Pont luciferase–luciferin reagent to be linear. Dilution of the mixed luciferase–luciferin reagents has a marked effect on light production unless the diluted reagent is supplemented with luciferin. The light production from a given quantity of ATP varies depending upon the relative luciferase and luciferin concentrations (6).

Luciferin. The effect of adding various concentrations of luciferin to a luciferase preparation that is completely luciferin-dependent is shown in Fig. 4. Most of the commercial reagents that contain luciferin are not saturated with luciferin (6). Karl and Holm-Hansen (14) have shown that additional luciferin increased the response
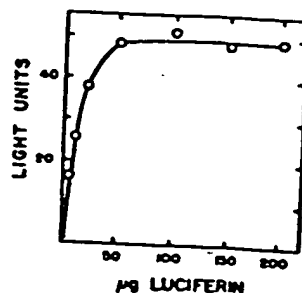

Fig. 4. Effect of luciferin concentration on light production. The reaction system (1 ml) contained 0.025 M Tricine buffer, pH 7.8, 2 μg Sigma Type IV luciferase, 1 ng ATP, 5 mM Mg²⁻, 0.5 mM EDTA, 0.5 mM DTT, and various amounts of luciferin.

TABLE II

Effects of Additions to or Omissions from the Reaction System on
Light Production by Firefly Luciferase[a]

	Activity (light units)		
	Sigma FLE-50	Du Pont	Sigma Type IV
Part A. Additions			
None	1.1	1.3	0.2
Mg^{2+}	1.6	1.7	0.1
EDTA	0.1	0.2	0.1
Luciferin	70.4	9.5	2.0
DTT	1.2	1.5	0.1
Bovine serum albumin	2.4	1.7	0
Part B. Omissions			
None	37.9	12.0	4.3
Mg^{2+}	19.9	1.1	0
EDTA	32.0	11.1	4.4
Luciferin	0.5	1.9	0
DTT	25.0	10.5	4.0
Bovine serum albumin	19.4	11.3	2.0

[a] Reactions were conducted in Bio-Vials using 100 pg of ATP, and 0.025 M Tricine buffer, pH 7.8, with peak height measurements of light emission. The amounts of luciferase used were: Du Pont, 400 μg; Sigma FLE-50, 400 μg; Sigma Type IV, 2 μg. The other reaction components were either added individually (Part A) or omitted individually (Part B) from a reaction mixture containing all of the basic components. The concentrations used in the final reaction mixture (1.0 ml) were: luciferin, 50 μg; bovine serum albumin, 100 μg; Mg^{2+}, 5 mM; EDTA, 0.5 mM; and DTT, 0.5 mM.

given by Sigma FLE-50 lantern extracts. They found that aging of the enzyme preparations was required to reduce the background. For greatest sensitivity in measuring ATP most commercial preparations require added luciferin.

Oxygen. Oxygen is a substrate in the luciferase-catalyzed reaction. Under normal assay conditions no stimulation was observed by bubbling oxygen through the Tricine buffer solution for up to 30 min before use. To demonstrate an oxygen requirement glucose and glucose oxidase were incubated with the luciferase reaction mixture prior to addition of ATP. Either of those two reagents alone reduced the light output only 7–9% while in combination they reduced light output by 77%. Under normal assay conditions oxygen was not limiting.

Effects of Other Additions to and Omissions from the Assay System. Three typical luciferase preparations were compared because they represented the three levels of purity commercially available: Sigma FLE-50, a crude lantern extract; Du Pont, a partially purified luciferase reagent; and Sigma Type IV, a crystalline luciferase. Table II, part A, shows the effect of various additives. The activity of the FLE-50 preparation was stimulated by Mg^{2+}, luciferin, and bovine serum albumin. Light production by the Du Pont luciferase was stimulated by luciferin. Single additions to the Sigma Type IV luciferase resulted in only slight increases because most of the required components were not present in the assay mixture. In each case EDTA by itself was inhibitory. The effects of omissions from reaction mixtures containing all of these components are shown in Table II, part B. All luciferase preparations had decreased activity without additional luciferin.

TABLE III

Reversal of Arsenate Inhibition of Firefly Luciferase[a]

Substance added	Light units	Percentage of control
Experiment A		
None	8.2	49
0.1 M Phosphate	2.7	61
400 μg Luciferase–luciferin	30.2	180
10 mM MgSO₄	7.5	45
10 ng Pyrophosphate	6.3	38
5 ng AMP	7.4	44
Experiment B		
None	34.4	46
2 μg Luciferase	91.9	123
50 μg Luciferin	37.3	50

[a] Reaction mixtures (1 ml) contained 0.025 M Tricine buffer, pH 7.8, 5 ng ATP, 0.01 M arsenate, 5 mM MgSO₄, 0.5 mM EDTA, and 0.5 mM DTT. Integrated counts were measured for a 60-s period after a 15-s delay. For Experiment A 400 μg of Du Pont luciferase–luciferin mixture was used and the arsenate-free mixture produced 16.8 light units, which was used as the control value. In Experiment B, 2 μg of Sigma Type IV luciferase and 50 μg of luciferin were used. The arsenate-free reaction produced 74.6 light units, which was used as the control value.

Because of the essential nature of the sulfhydryl groups (15) DTT is included in the complete reaction mixture. Bovine serum albumin was stimulatory with both Sigma preparations (reduced light output when omitted). Many of the partially purified luciferase preparations are supplemented with bovine serum albumin to increase stability. Mg^{2+} is required by all preparations.

Inhibitors

Ionic strength effects. Denburg and McElroy (8) showed that luciferase has one anion-binding site per active site and that all anions bind at the same site. The ionic strength effect increases the K_m for the magnesium ion–ATP complex. The effect

TABLE IV

Inhibition of Luciferase by Arsenite and 2,3-Dimercapto-1-propanol[a]

Buffer	Arsenite concentration (M)	Activity (light units)[b]	
		No addition	0.01 M BAL
Phosphate buffer, 0.025 M	0	118.5 [100]	38.9 [33]
	0.01	142.3 [120]	9.7 [8]
	0.1	68.3 [58]	5.1 [4]
Tricine buffer, 0.025 M	0	109.2 [100]	56.1 [51]
	0.01	87.2 [80]	25.9 [24]
	0.1	4.6 [4]	0 [0]

[a] Reaction mixtures (1 ml) contained the indicated buffer, 5 mM MgSO₄, 0.5 mM EDTA, 400 μg of Du Pont luciferase, and 50 ng of ATP.

[b] Values in brackets show the activity as percentage of control without added arsenite or BAL.

TABLE V

Effect of Volume and Cuvette Size on Measured Light Production[a]

Reaction volume (ml)	ATP (pg)	Luciferase (μg)	Light units		
			Scintillation vial	Beckman Bio-Vial	6 × 50-mm tube
1	1000	400	33.7	47.8	
0.5	500	200		27.6	14.3
0.5	100	200	3.0	5.8	1.8
0.2	100	200		26.7	16.8

[a] Du Pont luciferase–luciferin was used in the amounts indicated. The reaction system contained 0.025 M Tricine buffer, pH 7.8, 5 mM $MgSO_4$, 0.5 mM EDTA, and 0.5 mM DTT.

of arsenate on luciferase is not specific (18). Table III shows which component of the reaction mixture reverses the inhibition of light production by arsenate. Part A shows that an increase in the concentration of luciferase–luciferin reversed the inhibition. When pure luciferase and luciferin were used (Table III, part B), the luciferase component was active in reversing arsenate inhibition. This reversal would operate by increasing the number of binding sites for the anions.

Sulfhydryl reagents. DeLuca *et al.* (15) have shown that there are two essential sulfhydryl groups in luciferase. We found that dithiothreitol gave a 1.6-fold stimulation of light production while both mercaptoethanol and cysteine gave 1.1-fold stimulations. Dithiothreitol was effective in reversing *p*-chloromercuribenzoate inhibition. The existence of two essential sulfhydryl groups raises the question of their proximity; this was examined using arsenite. British anti-Lewisite is known to reverse arsenite inhibition specifically. When BAL was used in an attempt to reverse arsenite inhibition of luciferase, BAL itself was seen to be inhibitory and arsenite and BAL each potentiated the inhibition produced by the other compound (Table IV). Results are shown using both phosphate buffer and Tricine buffer for the assay of luciferase. We have shown previously that luciferase has different conformations depending upon which buffer is used (9).

REACTION AND MEASUREMENT CONDITIONS

Assay Volume

Table V shows results obtained using reaction vessels of different sizes and different reaction volumes in the SAI Model 3000 photometer. With a 1-ml reaction volume, change from a standard 25-mm-diameter glass scintillation vial to a 10-mm-diameter plastic vial increased the measured light output by 40%. A further reduction in the diameter of the reaction vessel to 6 mm, rather than further increasing the measured light output, decreased it by about 40%. A reduction of reaction volume from 0.5 to 0.2 ml in the Bio-Vial gave about a fivefold increase in the measured light output.

Temperature

The optimum temperature for light output was 25°C, as reported by McElroy and Strehler (17). At 30°C there was a 20% reduction in light output while at 20°C there was only a 5% reduction in light output.
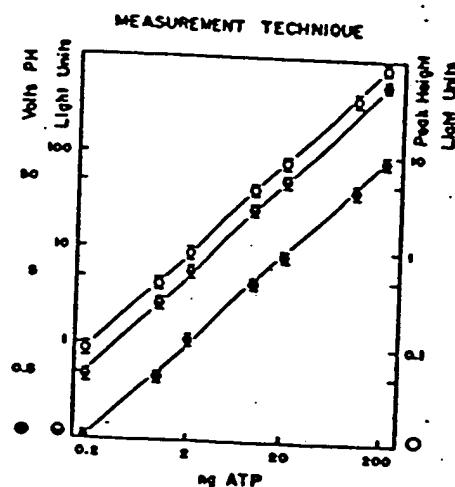
FIG. 5. Measurement of luciferase activity by peak height voltage, peak height counts, and integration of light production. The light output from reaction mixtures (1 ml) containing 0.025 M Tricine buffer, pH 7.8, 5 mM Mg^{2+}, 0.5 mM EDTA, 0.5 mM DTT, 400 µg of Du Pont luciferase–luciferin was determined.

Measurement Conditions

DeLuca *et al.* (18) suggested that integrating total light output for an arbitrary time could lead to serious errors in the determination of ATP content at different final concentrations of ATP. However, they also stated that no single method of measuring light production was adequate for all conditions used for ATP analysis. Therefore, we compared three methods for measuring light production using the SAI Model 3000 photometer. Figure 5 shows parallelism between the dependence of light output on ATP concentration when light output was measured by the peak height determined by recorder trace, by the counts when the photometer was used in the peak height mode (a 2-s delay followed by a 1-s count), and by counts determined in the integration mode (15-s delay, 60-s count).

STABILITY OF REAGENTS

For routine assays it is convenient to prepare stock solutions that can be stored. Experiments were done to define suitable conditions for storage and the length of time that the reagents remained useful.

Luciferase

The stability of luciferase in solution was measured for up to 50 days of storage (Table VI). The enzyme was completely stable in either phosphate or Tricine buffer for at least 24 h. There was little activity loss until 10 days after preparation. The enzyme preparations were useful for up to 3–4 weeks if ATP standards were used to correct for the loss of activity after 10 days. A sufficient amount of activity remained after 50 days for the detection of 1 ng of ATP.

Another way to store the luciferase was to freeze the preparation. Table VII shows that luciferase lost about 15% of its activity when thawed and refrozen on 4 consecutive days. After 4 weeks of storage in frozen state there was only a 15–25% loss and after 8 weeks of storage in this way there was 9–37% loss.

Spiegel and Tifft (18) observed 90% loss of luciferase activity when Du Pont

TABLE VI

Stability of Luciferase on Storage[a]

Time	Residual activity (%)	
	Tricine buffer	Phosphate buffer
1 hr	95	86
3 hr	90	96
5 hr	106	101
24 hr	102	111
2 days	104	115
4 days	109	109
7 days	94	115
10 days	96	120
14 days	75	104
21 days	57	86
28 days	63	—
49 days	22	65

[a] Du Pont luciferase–luciferin in Tricine or phosphate buffer was stored for the indicated time at 4°C. The enzymatic activity was determined in a 1-ml reaction system containing 10 ng ATP, 0.025 M Tricine, pH 7.8, 5 mM Mg^{2+}, 0.5 mM EDTA, and 0.5 mM DTT.

luciferase–luciferin reagent preparations were stored refrigerated in the dark for 4 days. The preparation was stable for 4 days if stored frozen and in the dark.

ATP

ATP solutions of concentration 5 ng/ml were prepared in Tris, phosphate, Mops, and Hepes buffers, and the light output each produced was measured at various

TABLE VII

Stability of Luciferase on Freezing and Thawing[a]

Time (days)	Residual activity (%)	
	Du Pont	Sigma Type IV
Repeated freeze-thaw treatment		
2	80	81
3	75	78
4	80	92
Single freeze-thaw treatment, different lengths of storage		
7	73	75
14	75	81
28	74	85
56	63	91

[a] The Du Pont luciferase–luciferin and the Sigma Type IV luciferase were stored at −15°C. The same sample was frozen and thawed each day for the results in the upper part of the table. The results in the lower part of the table were obtained with individual samples thawed for test after the appropriate storage period. The reaction system (1.0 ml) contained 5 ng ATP, 0.025 M Tricine, pH 7.8, 5 mM Mg^{2+}, 0.5 mM EDTA, and 0.5 mM DTT. With the Sigma Type IV preparation luciferin/ 50 µg was added.
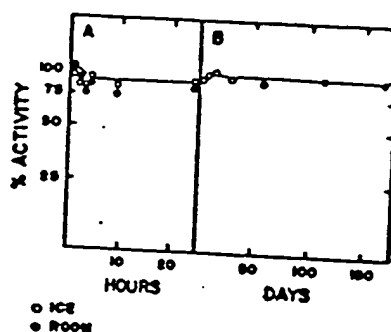
WEBSTER AND LEACH

FIG. 6. Stability of luciferin upon storage. Luciferin (5 mg/ml) was dissolved in sterile water and stored in Beckman Bio-Vials wrapped with aluminum foil and flushed with nitrogen. The reaction system (1 ml) contained 0.025 M Tricine, pH 7.8, 1 ng ATP, 5 mM Mg^{2+}, 0.5 mM EDTA, 0.5 mM DTT, and 400 μg of Du Pont luciferase–luciferin. A 12-fold increase in light output is produced by the additional 50 μg of luciferin. For (A) the luciferin was stored on ice (4°C) and at room temperature (20°C). For (B) the luciferin was stored frozen.

times during a 26-h storage period at room temperature (22–25°C). The solutions in Tris, Mops, and Hepes buffers were completely stable for 6 h after which time a slight drop occurred. The stability in phosphate buffer was less. Stock solutions of ATP of concentration 10 μg/ml or greater can be stored in deionized water or Tris buffer without serious loss for at least a year if kept frozen and sterile. Spiegel and Tifft (18) found no problems with the stability of ATP standards during a 4-day period.

Luciferin

When luciferin preparations (concentration 1 mg/ml) are stored either on ice or at room temperature (21°C) and stored under N_2 in a tightly capped vial, there is little loss of luciferin (Fig. 6A). Figure 6B shows that luciferin at 5 mg/ml is stable for 24 weeks when stored frozen (−15°C) under nitrogen.

REFERENCES

1. BORUN, G. A. (ed.) (1975) ATP Methodology Seminar, SAI Technology Co., San Diego, Calif.
2. BORUN, G. A. (ed.) (1977) 2nd Bi-Annual ATP Methodology Symposium, SAI Technology Co., San Diego, Calif.
3. SCHRAM, E., AND STANLEY, P. (eds.) (1979) International Symposium on Analytical Applications of Bioluminescence and Chemiluminescence, State Printing and Publishing, Inc., Westlake Village, Calif.
4. DELUCA, M. A. (ed.) (1978) in Methods in Enzymology, Vol. 57, Academic Press, New York.
5. HERRING, P. J. (ed.) (1978) Bioluminescence In Action, Academic Press, New York.
6. WEBSTER, J. J., CHANG, J. C., HOWARD, J. L., AND LEACH, F. R. (1979) J. Appl. Biochem. 1, 471–478.
7. DENBURG, J. L., AND McELROY, W. D. (1970) Arch. Biochem. Biophys. 141, 668–675.
8. SELIGER, H. H., AND McELROY, W. D. (1960) Radiat. Res. Suppl. 2, 528–538.

9. WEBSTER, J. J., CHANG, J. C., MANLEY, E. C., SPIVEY, H. O., AND LEACH, F. R. (1980) *Anal. Biochem.* 106, 7–11.

10. STREHLER, B. L., AND TOTTER, J. R. (1952) *Arch. Biochem. Biophys.* 40, 28–41.

11. McELROY, W. D. (1977) *in* 2nd Annual Methodology Symposium (Borun, G. A., ed.), pp. 405–413, SAI Technology Co., San Diego.

12. WILLIARD, J. M., DAVIS, J. J., AND WOOD, H. G. (1969) *Biochemistry* 8, 3137–3144.

13. GREEN, A. A., AND McELROY, W. D. (1956) *Biochim. Biophys. Acta* 20, 170–176.

14. KARL, D. M., AND HOLM-HANSEN, O. (1976) *Anal. Biochem.* 75, 100–112.

15. DELUCA, M., WERTZ, G., AND McELROY, W. D. (1964) *Biochemistry* 3, 935–939.

16. DELUCA, M., WANNLUND, J., AND McELROY, W. D. (1979) *Anal. Biochem.* 95, 194–198.

17. McELROY, W. D., AND STREHLER, B. L. (1949) *Arch. Biochem. Biophys.* 22, 420–433.

18. SPIEGEL, S. J., AND TIFFT, E. C. JR. (1977) *Anal. Biochem.* 78, 586–588.